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(54) Title: IMMUNOTHERAPEUTIC AGENTS, COMPOSITIONS AND METHODS

(57) Abstract

The invention provides a pharmaceutical preparation for specifically altering the immune response of a mammal, comprising mononuclear leukocytes form said mammal, which leukocytes have been treated by extracorporeal incubation with an agent which induces irreversible damage to the cells or cell death. The invention also provides a pharmaceutical composition and a method for treatment of an immunologically-mediated condition or a cancer, and a method of preparation of a pharmaceutical preparation according to the invention.

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IMMUNOTHERAPEUTIC AGENTS, COMPOSITIONS AND METHODS

This invention relates to methods of treatment of immunologically related conditions, and to agents and compositions thereof. In particular, the invention relates to a method of treatment of the patient's own lymphocytes, which treatment is carried out extracorporeally.

Abbreviations

Abbreviations used herein are as follows: UVA ultraviolet A light, 8-MOP 8-methoxypsoralen, dCF deoxycoformycin, dAdo deoxyadenosine, NOD mouse non-obese diabetic mouse, CTCL cutaneous T-cell lymphoma, PBL peripheral blood lymphocytes, ECPC extracorporeal photochemotherapy, CP cyclophosphamide, Con A Concanavalin A EDTA ethylenediaminotetraacetate, BSS mouse isotonic buffered balanced salt solution; PBL peripheral blood lymphocytes; PBS phosphate buffered saline; %D % double-stranded DNA remaining; DNA SSBs DNA single-strand breaks; MDSS maximum decrease in skin score; S.E. standard error.

Background of the Invention

Treatments to augment the activity of lymphocytes, in particular T cells, have been proposed as a means of treatment for autoimmune disease, and it has been suggested that such treatments result in the generation of 'anti-clonotypic' T cells which inhibit the patient's autoreactive T cells. The procedure has been termed 'T cell vaccination' (Cohen and Weiner, 1988). The most widely clinically investigated of these procedures is known as photopheresis; this involves exposure of cells to a psoralen, followed by photoactivation with ultraviolet A. See, for example, European Patent Application No. 261648 and Australian Patent Application No. 13584/88, the entire disclosures of which are herein incorporated by reference. It is evident that the following procedures of extracorporeal treatment of lymphocytes are known or suggested to be effective in the treatment of autoimmune

disease and/or cancer:

Raised hydrostatic pressure;

Chemical cross linking, eg. with formaldehyde or glutaraldehyde;

Photoactivated cross-linking using a psoralen and ultraviolet A, optionally with prior activation of lymphocytes using eg. Con A;

Activation of lymphocytes eg. with Con A, followed by chemical cross-linking;

Irradiation;

Mitomycin C;

Very high or low temperature;

High or low pH;

Low pressure;

Chemicals or toxins; and

Passage through resinous materials.

It appears that at least some of these treatments, in particular psoralen plus ultraviolet A, require prior activation of target lymphocytes by immunization of the subject to be treated, or by activation of cells in vitro, before treatment. See, for example, 13584/88.

Infusions of photoirradiated T-cells prevent or ameliorate a number of diseases in animal models of autoimmune disease and transplantation (Knobler and Edelson, 1986; Anderson and Voorhees, 1980). In humans, extracorporeal photochemotherapy (ECPC) is being investigated as a treatment for cutaneous T-cell lymphoma, systemic lupus erythematosus, type I diabetes and other diseases mediated by T-lymphocytes (Gilchrest et al, 1976; Edelson et al, 1987). A major therapeutic potential may be for autoimmune diseases.

Cutaneous T-cell lymphoma (CTCL) is a malignant monoclonal proliferation of T-lymphocytes, usually those of the helper phenotype (Kubler and Edelson, 1986). The diseases encompassed include Sezary syndrome, mycosis fungoides and various adult T-cell leukemias. There are up to 10,000 new cases per year in the USA; it is more common in males, and is usually diagnosed after the age of 40. It

typically remains localized to the skin for a time, then evolves into a nonepidermotropic stage in which there is dissemination and involvement of other organ systems. Extracutaneous spread is associated with refractoriness to treatment and a poor prognosis.

Therapy for disseminated CTCL includes systemic chemotherapy, leukapheresis and the use of monoclonal antibodies directed against the malignant lymphocyte phenotype. Oral 8-MOP photochemotherapy (PUVA) is effective in early stage skin-localized CTCL (Anderson and Voorhees, 1980; Gilchrest et al, 1976). It produces few side-effects, does not cross-react with other treatments and results in relatively long-lived remissions. Its effects are limited to tissues exposed to UVA because of the short half life of the photoactive form of the drug.

In 1987, Edelson and colleagues described 37 patients with CTCL treated with ECPC. In this technique, following an oral dose of 8-MOP, patients were leukapheresed and their buffy coat lymphocytes extracorporeally exposed to UVA light for 3 hours, and then reinfused into the donor patient. 73% of all patients responded with a mean of 64% decrease in cutaneous involvement (skin score). Their median survival is now in excess of 4 years (fewer than 50% of the 37 patients are now dead), greater than that for historical controls (Edelson et al, 1987). However, although it shows promise of clinical effectiveness and is being investigated in other conditions, ECPC is cumbersome, inconvenient and expensive.

The mechanism of action of ECPC in CTCL is not well understood. There is evidence that the damaged lymphocytes incite an immune reaction when they are returned to the patient. In vitro studies showed that about 90% of these irradiated lymphocytes are non-viable at 96 hours (Edelson et al, 1987). Detailed studies of the mechanism of this cytotoxicity have not been performed, but it is presumed that the DNA damage caused by photoactivated psoralens is the mechanism of cell death. DNA-damaging agents may kill cells by causing ATP and NAD depletion

secondary to poly (ADP-ribosyl)ation at sites of DNA strand breakage (Berger, 1985): this might be an important mechanism of the lymphocytotoxicity of CTCL cells treated with ECPC.

We report herein studies with treated patients' buffy coat lymphocytes, complemented by in vitro experiments with PBL using a UVA light source and 8-MOP. We show the potentiation of UVA damage to lymphocyte DNA by 8-MOP and the correlation of cytotoxicity with subsequent adenine nucleotide depletion, apparently secondary to poly (ADP-ribosyl)ation. We have now found that the lymphocytotoxic effect of ECPC in CTCL is mediated by DNA damage, with concomitant stimulation of poly (ADP-ribose) synthetase and depletion of adenine nucleotides in the cells. The treated cells appear programmed to die by apoptosis, with characteristic morphological changes associated with endonucleolytic DNA cleavage.

We have now investigated, by in vitro studies with normal human PBL and in an animal model, whether the effects of UVA/8-MOP extracorporeal treatment of lymphocytes could be mimicked by treatment with a cytotoxic agent. The test system selected was dCF/dAdo, which has been well characterised. The combination of dCF and dAdo caused similar biochemical and physical effects to UVA/8-MOP in human PBL. CP-induced diabetes in the NOD/WEHI mouse was chosen as an animal model, because it provided a well-defined autoimmune disease with clear, easily assessed endpoints (Bescher et al, 1990; Yasunumi and Bach, 1988; Harada and Makino, 1984).

We now report that the infusion of syngeneic mouse splenocytes treated with UVA/8-MOP significantly reduces the incidence of diabetes in CP-treated 90-120 day old NOD/WEHI mice. There was a similarly low incidence of diabetes in a group of mice that received splenocytes treated with dCF and dAdo. Both forms of treatment induce apoptosis in these splenocytes.

Summary of the Invention

According to one aspect of the present invention there is provided a pharmaceutical preparation for

specifically altering the immune response of a mammal, comprising mononuclear leukocytes from said mammal, which leukocytes have been treated by extracorporeal incubation with an agent which induces irreversible damage to the cells or cell death.

In a second aspect, the invention provides a pharmaceutical composition for treatment of an immunologically-mediated condition or a cancer, comprising an effective amount of a preparation according to the first aspect of the invention, together with a pharmaceutically-acceptable carrier.

In a third aspect, the invention provides a method for prevention or treatment of an immunologically-mediated condition or of a cancer, comprising the step of administering to a patient in need of such treatment an effective quantity of a composition according to the second aspect of the invention.

In a fourth aspect, the invention provides a method of preparation of a pharmaceutical preparation according to the invention, comprising the steps of incubating a sample of cells from a patient suffering from an immunologically-mediated condition or a cancer, said sample comprising normal or malignant mononuclear leukocytes, with an agent which induces irreversible damage to the cells or cell death, for a period and under conditions effective to cause irreversible damage to said cells, and optionally washing the cells.

Agents suitable for use in the invention are those which damage cellular DNA. Suitable agents for use in the invention include, but are not necessarily limited to antineoplastic or antiviral agents, especially nucleoside analogues, alkylating agents, inhibitors of topisomerase-II, antimetabolites and anthracyclines, and steroids. Two or more active agents may be used in combination. Particularly preferred agents are as follows:

1. An adenosine deaminase inhibitor in combination with an adenosine analogue. Preferred adenosine deaminase inhibitors are deoxycoformycin, EHNA (erythro-9-(3-hydroxy-

2-nonyl)adenine, and 2-chlorodeoxyadenosine, while preferred adenosine analogues are 2'-deoxyadenosine, 3'-deoxyadenosine (cordycepin), tubercidin and 9-(β-D-arabinofuranosyl)adenine (Ara-A; vidarabine);

2. Alkylating agents; preferred alkylating agents are mafosfamide and 4-hydroperoxycyclo-phosphamide;
3. Antimetabolites; a preferred antimetabolite is methotrexate;
4. Inhibitors of topoisomerase II; preferred inhibitors are etoposide (VP-16-213) and teniposide (VP-16), and other derivatives of podophyllotoxin; and
5. Antibiotics which induce DNA strand breakage, such as the bleomycins, mitomycin, anthracyclines such as daunorubicin and epirubicin, and gliotoxin.

It is also to be clearly understood that analogues or derivatives of the specific agents named which may also be used, provided that they are effective in inducing cell death. Such effectiveness may be readily established by methods known in the art.

The cells which are the target of the extracorporeal treatment will normally have been activated in vivo as part of the disease process. However, the method of the invention may also optionally include a specific step of activation of the target cells prior to extracorporeal treatment, either by immunization of the subject or by treatment of the cells in vitro with an agent such as Con A. Such a step would have the advantage of expanding the clone of activated target cells which are to be treated.

It is considered to be a matter of routine experimentation for a person skilled in the art to establish appropriate concentrations of active agents, and a time of treatment which is convenient and practicable for clinical use, bearing in mind that it is desirable to

minimise the time of incubation in order to avoid infection.

The cells to be treated are normal or malignant mononuclear leukocytes of lymphocytic or monocytic lineage, preferably lymphoid cells. These cells may be obtained from blood, lymph, bone marrow, or lymphoid tissue. For convenience, the preferred cells to be treated are buffy coat leukocytes.

The method of the invention is suitable for the treatment of a condition selected from the group consisting of:

autoimmune diseases;
cancer;
allograft and/or xenograft rejection;
graft-versus-host disease;
delayed-type hypersensitivity; and
allergy,

especially autoimmune diseases, and malignancies of the lymphoid system.

Without wishing to be bound by any proposed mechanism for the observed beneficial effects, the treatment of the invention results in the induction of cell death; it is thought that the treatment of the invention induces programmed cell death (apoptosis) rather than necrosis. Thus agents which induce apoptosis are particularly preferred for use in the invention. Such agents induce fragmentation of cellular DNA, and include bleomycin, anthracyclines and gliotoxin.

Brief Description of the Invention

The invention will now be illustrated by way of reference only to the following non-limiting examples, and to the drawings, in which:

Figure 1 shows % increase in DNA single strand breaks vs. % decrease in cellular (trypan blue) viability. Simple regression analysis yields a R^2 of 0.877. The intercept for the graph is 82.1 (38.9-125.2, 95% confidence limits) and the gradient is -1.03 (-2.12-0.06).

Figure 2 shows ffects on viability. A. PBL

were exposed to UVA light (1-2 J/cm^2) with or without 8-MOP (300 ng/ml) and their corrected viability determined by trypan blue exclusion every 24 hours. A. 1 J/cm^2 UVA, B. 1 J/cm^2 UVA + 8-MOP, C. 2 J/cm^2 UVA, D. 2 J/cm^2 UVA + 8-MOP. Mean of triplicate samples from a representative experiment (with multiple replicate experiments).

B. PBL were exposed to UVA light (2 J/cm^2) and 300 ng/ml 8-MOP with or without 2 mM 3-ABA and corrected viability was determined at 24 and 48 hours. This is compared with incubating PBL with 2 mM 3-ABA alone. A. 3-ABA alone, B. 2 J/cm^2 UVA + 8-MOP, C. 2 J/cm^2 UVA + 8-MOP + 3-ABA. The results represent the results (performed in triplicate) from a typical sample. Multiple replicate experiments show the same pattern. The two figures represent experiments involving PBL from different donors. Control lymphocyte viability was 92%.

Figure 3 shows DNA damage in PBL exposed to UVA light (1-5 J/cm^2) with or without 8-MOP (300 ng/ml); the % increase in DNA single strand breaks was measured 2 hours later fluorometrically. Mean of 2 experiments each performed in duplicate.

Figure 4 shows effects on nucleotide content.

A. PBL were exposed to UVA light (1-40 J/cm^2) then 2 hours later extracted with perchloric acid and their NAD and ATP content measured by HPLC. Error bars indicate standard error of 3-4 observations.

B. PBL were exposed to UVA light (2 J/cm^2) in the presence of 300 ng/ml 8-MOP with (B) or without 2 mM 3-ABA (A), and 2 hours later their NAD and ATP content (% control) were measured. Error bars indicate the standard error of 3-4 observations.

Figure 5 shows a comparison between photochemotherapy and treatment with deoxycoformycin plus deoxyadenosine.

A. PBL were exposed to 1 μM dCF and 0-20 μM dAdo for 4 hours and corrected viability was determined for the next 72 hours. A. 1 μM dCF, B. 1 μM dCF + 5 μM dAdo, C. 1 μM dCF + 10 μM dAdo, D. 1 μM dCF + 20 M dAdo. Mean of 3

observations.

B. Two hours after a 4 hour exposure to 1 μM dCF and 10 μM dAdo, cellular NAD and ATP levels were determined (mean of two experiments). These are compared with the nucleotide depletion of lymphocytes caused by ECPC.

Figure 6 shows results of an experiment in which mouse splenocytes were exposed to various cytotoxic agents and their corrected viability measured by trypan blue exclusion for the next 48 hours. A. 1: 1 J/cm^2 UVA light. 2: 1 J/cm^2 UVA light plus 100 ng/ml 8-MOP. B. 1 μM dCF, 5 μM dAdo for 4 hours. C. 50 μM mafosfamide for 1 hour. D. 10 μM mitomycin C for 1 hour. Results represent the mean of at least 2 separate experiments preferred in duplicate.

Figure 7 shows the incidence of diabetes (%) in CP-treated NOD/WEHI mice whose splenocytes were subjected to various treatments. Recipient NOD/WEHI mice were given 350 mg/kg intraperitoneal CP on day 1 and had their plasma glucose measured 14-18 days later. Diabetes was defined as a plasma glucose level of 15 mM or more. There were 5 treatment groups: no further treatment (0); untreated mouse splenocytes (untreated); mouse splenocytes treated with 1 J/cm^2 UVA (UVA only); mouse splenocytes treated with 1 J/cm^2 UVA in the presence of 100 ng/ml 8-MOP; mouse splenocytes treated with a 4 hour exposure to 1 μM dCF and 10 μM dAdo.

Materials and Methods

Photophoresis procedure:

All patients were treated with the UVAR photopheresis system (Therakos, King of Prussia, PA, USA). This machine is the vehicle for the collection of buffy coat lymphocytes and also contains the clear plastic disposable cassette where lymphocytes are exposed to UVA light. At 0 hours, the patients ingested 8-MOP tablets at a dose of 0.6 mg/kg. One and a half hours later, the patients were venepunctured and connected to the photopheresis machine. Fifteen minutes later, when the first buffy coat sample (40 ml) had been collected, the UVA

source was switched on to irradiate the buffy coat sample. Over the next 1.5-2 hours (depending on the rate of venous blood flow) a further 5 buffy coat samples were collected. In total, there were 6 cycles of blood withdrawal, centrifugation and reinfusion. Red cells and all additional plasma were returned to the patient before beginning the next collection cycle. At this stage (3.25-3.75 hours after tablet ingestion) the final volume for photoirradiation was reached and the patient was disconnected from the machine. The final volume of 740 ml consisted of 240 ml buffy coat, 300 ml plasma and 200 ml heparinised normal saline. Photoactivation continued for another 1.5 hours. The hematocrit of the leucocyte-enriched blood was always less than 7% because red cells block leucocyte absorption of UV energy. The mean number of cells reinfused was 3.1×10^9 (range $1.26\text{-}4.9 \times 10^9$) which is about 25% of the total circulating numbers of lymphocytes.

Treatment policy and assessment of response:

Patients were treated on two consecutive days every 4 weeks for 6 months, with treatment frequency modified according to clinical response. Disease progress was monitored by measurement of standardized skin scores, skin biopsies and by regular clinical photographs. Briefly, all skin was graded from 0 to 4, with 4 representing the most severe disease. This was then multiplied by the percentage of total surface area involved so the maximum possible score is 400. Skin score assessments were made before the commencement of therapy and then monthly before each course of ECPC by an experienced dermatologist. Response was defined as a 25% improvement in the skin score, sustainable over a one month period. Complete reassessment of all patients occurred 6 months after starting treatment.

Processing of buffy coat specimens:

Specimens of buffy coat blood were obtained from

the first buffy coat specimen and also after UV irradiation was complete, immediately before reinfusion to the patient. About 20 ml of buffy coat blood was underlaid with 5 ml of Ficoll-Hypaque and spun at 450g for 25 minutes. The cloudy mononuclear cell fraction was removed and counted. After washing in phosphate-buffered saline, the lymphocytes were resuspended in RPMI 1640 plus 10% fetal calf serum. After the above handling, the mean composition of the cells (± S.E.) was: lymphocytes 74 ± 9%, granulocytes (including band forms) 22 ± 8% and monocytes 3 ± 1%.

Viability:

For viability studies, lymphocytes from the buffy coat samples were suspended in RPMI 1640 plus 10% fetal calf serum and incubated at 37°C. Viability was determined by the ability of cells to exclude 0.5% trypan blue. Viability was assessed every 24 for 96 hours after photoirradiation and compared with the 24-96 hour viabilities of the unirradiated buffy coat lymphocytes which were similarly handled.

Measurement of DNA single-strand breaks:

About 1 x 10^7 lymphocytes were centrifuged to form a pellet and then processed in triplicate samples according to the fluorometric method of Birnboim and Jevcak (1981). The percentage of double-stranded DNA remaining (% D) of the first (control) specimen was corrected to 100 and the % D value obtained for the second specimen was expressed as a percentage of this. The increase in DNA SSBs was calculated by subtracting the % D value from 100. The *in vitro* processing of lymphocytes from normal human donors and their exposure to 8-MOP did not affect cellular viability; viability was unimpaired at the time of measuring DNA damage.

Measurement of NAD and ATP content:

Approximately 2 x 10^7 lymphocytes were centrifuged to form a pellet, extracted with ice-cold perchloric acid and their NAD and ATP content were measured

by HPLC as previously described (Crescentini and Stocchi, 1984). The lymphocytes obtained from the unirradiated buffy coat specimen were regarded as having normal (control) NAD and ATP content and the nucleotide content of the lymphocytes from the second specimen was calculated as a percentage of this control value.

Normal human peripheral blood lymphocytes:

Fresh human peripheral lymphocytes were obtained by venesection or from normal human volunteers' buffy coat specimens (Victorian Red Cross Blood Bank) which were available within 1 hour of leukapheresis. The 40 ml buffy coat specimen was overlaid onto 5-10 ml of Ficoll-Hypaque and spun at 450g for 25 minutes. Two washes in phosphate-buffered saline were followed by suspension in RPMI 1640 plus 10% fetal calf serum and antibiotics.

UV irradiation of lymphocytes or splenocytes:

For UV irradiation, cells were suspended in culture medium at about 5 x 10^5 cells/ml. 8-MOP was freshly made up from stock solutions (1 mg/ml) and was added to cells shielded from the light for at least 15 minutes before photoirradiation. 5-10 ml aliquots of cell suspension were irradiated in 25 cm^2 plastic tissue culture flasks (Costar, Cambridge, MA, USA) with a Therakos research light box which contained a Photosette-R UVA light assembly mimicking the therapeutic equipment used for humans. Two banks of 6 lamps were located behind windows of transparent glass 10 cm apart. The lamps were operated at the maximum power setting (ten) for at least 10 minutes before experiments. The CSIRO National Measurement Laboratory of Australia calibrated the machine; a 32 second exposure was equivalent to 1 J/cm^2 of energy. The centre of the panel exclusively was used for UV irradiation.

Measurement of cellular poly (ADP-ribose) synthetase activity:

Poly (ADP-ribose) synthetase levels were measured

by the method of Berger et al, in which cells were rendered permeable to an exogenously supplied nucleotide and then the incorporation of ^{3}H-NAD into acid-precipitable material at 30°C was measured.

HPLC measurement of serum 8-MOP levels:

Serum 8-MOP levels were measured according to the HPLC method of Puglisi et al (1977). Blood samples were taken from all patients at hourly intervals during the period of photopheresis, so the peak 8-MOP level and its timing could be determined.

Non-obese diabetic mice:

The non-obese diabetic (NOD) mouse is a model of type I diabetes (Makino et al, 1980; Baxter et al 1990). A lymphocytic insulitis causes beta cell destruction with consequent hypoinsulinemia and hyperglycemia. Almost all NOD/WEHI mice have insulitis, but only a small proportion of susceptible mice (10% females and <1% males) spontaneously develop diabetes by 150 days of age. It is hypothesized that active suppression mechanisms prevent the progression from insulitis to diabetes.

CP, usually an immunosuppressive agent, appears to augment the development of diabetes in NOD/WEHI mice: a dose of 300-350 mg/kg increases the incidence of diabetes to nearly 70% within 21 days. The transfer of syngeneic mononuclear cells from prediabetic mice prevents CP-induced diabetes; the same cells from diabetic mice do not affect the incidence of diabetes. This suggests that CP has an immunological mechanism of action; it does not directly damage beta cells, nor does it cause diabetes in any of several other strains of mice not prone to develop diabetes. The mechanism by which CP increases the frequency of diabetes is unresolved, but there appear to be suppressor mechanisms operating to slow the damage of insulitis, thereby preventing diabetes; CP seems to negate these suppressors (Charlton et al, 1989; Harada and Makino,

1984).

Mice were maintained in specific pathogen-free conditions before being placed in an open animal room where they were fed commercial food pellets and water ad libitum. All mice were maintained on racks in the same room, with a 12 hour/12 hour light and dark cycle in a constant temperature of 21°C. Overtly unwell mice or mice with evidence of diabetes before treatment were excluded from experiments.

CP was freshly dissolved in mouse cell isotonic buffered balanced salt solution (BSS) and injected intraperitoneally into 100-120 day old female mice on day 1 at a dose of 350 mg/kg. This treatment causes diabetes in about 60% of mice 14 days later.

Pretreatment characteristics of donor and recipient mice and exclusion criteria:

Before entry into the study, recipient mice had to be shown to have a random plasma glucose of <12 mM. Diabetes was defined as a random plasma glucose of 15 mM or more. Although the incidence of spontaneous diabetes at 90-120 days of age is low (<5%), in relatively small treatment groups it could be significant; pretreatment glucose measurements eliminated this possibility.

Processing of mouse splenocytes:

Syngeneic mouse splenocytes were obtained from diabetic mice of approximately 110 days of age, 14 days after they had been given CP. Mice were killed by exposure to CO_2 and their spleens removed aseptically and placed in ice-cold BSS. The spleen was then processed by routine methods. Splenocytes were suspended in 0.156 M ammonium chloride, 0.1 mM EDTA and 12 mM sodium bicarbonate, pH 7.3, (a red cell lysis buffer) and left on ice for 5 minutes before resuspension in Dulbecco's modified Eagle medium with 5% fetal calf serum. One spleen yielded approximately 10^8 cells whose viability exceeded 80% by trypan blue exclusion.

Culture of mouse splenocytes and exposure to DNA-damaging agents:

Cells were washed and suspended in Dulbecco's modified Eagle medium with 5% fetal calf serum at a density of 10^6/ml before exposure, and the cytotoxic drugs were directly added. Cells were then washed in BSS and resuspended in 0.2 ml of this before injection into mice. During cytotoxic exposure, cells were maintained at 37°C in humidified conditions with 7% CO_2/93% air.

Plasma glucose measurement.

Tail vein blood was centrifuged at 10,000g for 10 minutes and the plasma removed. Plasma glucose estimations were made with a modified glucose oxidase technique on an autoanalyzer (Beckman Glucose Analyzer 2, Fullerton, CA, USA).

Light microscopy:

After cytotoxic exposure, cells were washed twice in RPMI 1640, and then pelleted (200g, 5 minutes). Cell specimens were fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate, resuspended in 1:1 horse serum:saline for 2 hours (4°C), then stained with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Each slide had several cell clumps taken from different areas in the pellet; cells were scored in several areas in order to avoid possible biases after centrifugation due to the differential density of cells. At least five hundred cells were counted per specimen.

Statistical analysis.

All p values were calculated using a chi- squared analysis of 2 x 2 contingency tables.

Materials:

Dulbecco's modified Eagle medium and Earle's basic salt solution were from Flow Laboratories (North Ryde, Sydney, Australia). 8-MOP, dAdo and mitomycin C were from Sigma (St Louis, MO) and cyclophosphamide was from

Farmitalia Carlo Erba (Hawthorn, Victoria, Australia). Deoxycoformycin was a gift from Parke-Davis (Morris Plains, NJ) and mafosfamide (Asta Z 7654) was from Asta Werke (Bielefeld, Germany). Aqueous lqiuid scintillant and ^{3}H-NAD(3Ci/mmol) were from Amersham (Bucks, U.K.). All other chemicals were from BDH (Kilsyth, Victoria, Australia).

Details of the materials for HPLC measurement of nucleotides and the fluorometric assay of DNA SSBs have been described[2] previously.

Examples 1 and 2 are comparative examples designed to investigate the mechanism whereby ECPC exerts its effect.

Example 1

Clinical response to extracorporeal photochemotherapy:

Four patients with CTCL (3 males and 1 female, mean age of 69) were treated with ECPC. Patients received 16-25 courses (mean 19) over a mean period of 25 weeks. One patient died of myocardial infarction and pulmonary embolism 7.5 months after the commencement of treatment. The 4 patients had significant palliation of their disease. The mean maximum decrease in skin score was 41% (range 29-55%, Table 1).

Effects of extracorporeal photochemotherapy on lymphocyte viability.

To assess the effect of the ECPC procedure on the patients' harvested lymphocytes, trypan blue viability was studied daily for 4 days following the procedure. This was studied in nine patient samples (from nine different treatment cycles) from the four patients. Viability progressively declined over several days, at a rate similar to that reported by Edelson et al (1987). Fewer than 5% of the treated cells were viable at 96 hours (Table 2).

DNA single-strand breaks.

To explore the mechanism of action of the ECPC-induced in vitro lymphocytotoxicity, DNA damage was

assessed. Although other forms of DNA damage may have been induced by the UVA/8-MOP, DNA single strand breaks were assayed by the fluorometric technique. DNA damage was assessed in samples of buffy coat lymphocytes after photoirradiation, just before reinfusion into the patient. The viability of photoirradiated cells at the time of measuring DNA SSBs was the same as that of non-photoirradiated control cells (85%). The control % double stranded DNA remaining (% D) value for non-photoirradiated lymphocytes was corrected to 100% and the treated lymphocyte sample expressed as a percentage of this. The absolute control % D value for the patients' cells (and for normal PBL) was 79 (range 60 to 97). All samples assessed had a marked increase in DNA SSBs (Table 1), but the extent of damage varied considerably between different patients, ranging from 9 to 60%. DNA damage was assessed twice in all patients except number 3. There was less than 15% variation between readings from samples at different time points; only the mean is stated. There was no correlation between the number of malignant cells and the percentage of DNA SSBs.

Adenine nucleotide content:

In view of the apparent association between DNA damage and lymphocytotoxicity, the extent of ATP and NAD depletion, possibly secondary to poly (ADP-ribosyl)ation, was investigated. Eight patient samples of immediate post-irradiation buffy coat lymphocytes (8 treatment cycles) from 4 different patients were analyzed. NAD content was measured in 3 of the 4 patients. All lymphocyte samples assessed showed depletion of nucleotides. The decline in NAD content was much more marked than the fall in ATP content (Table 1).

Table 1

Patients with CTCL: Clinical and Biochemical Data

Patient Number	Initial skin ATP viability	Minimum skin score content+	MDSS score	Mean Increase (%) content	24 hour % DNA SSBs	Mean % NAD (%)	Mean %
1	253	154	39	9	71	ND*	48
2	345	208	40	32	44	24	66
3	336	237	29	60	16	33	83
4	181	81	55	39	53	44	34
Mean	279	170	41	35	46	34	58

* not determined.

+ The NAD and ATP contents of buffy coat cells were measured immediately after photoirradiation.

DNA SSBs were measured immediately after photoirradiation by the fluorometric unwinding method (see methods).

The fall in the nucleotide content of patients' lymphocytes did not correlate with the degree of DNA damage to them. Absolute control NAD and ATP content (in patients' and normal PBL) were 60 (48-74) and 202 (180-220) pmol/10^6 lymphocytes respectively.

Correlation of biochemical parameters with clinical response: The biochemical and physical damage to buffy coat lymphocytes demonstrated above from CTCL patients has not been previously documented nor correlated with the clinical response of CTCL to ECPC. Four patients with CTCL were evaluable for response (Table 1). This number of patients precludes a meaningful statistical analysis of the relationship between response and the parameters of cell injury or death. However, the percentage of DNA SSBs was strongly associated with the decline in cellular viability (simple regression analysis, R^2=0.877, Figure 1). There was no correlation between the number of cells reinfused into a patient and that patient's clinical response (MDSS; data not shown)

Serum 8-MOP levels:

The 8-MOP levels of all patients were found to exceed 50 ng/ml during the entire period of photopheresis. The mean peak level was 168 ng/ml and occurred at an average of 2 hours after ingestion. Peak serum 8-MOP levels did not correlate with clinical response, in vitro lymphocytotoxicity or with any of the biochemical parameters associated with cell death.

Example 2

In vitro experiments on the mechanisms of 8-MOP and UVA phototherapy-induced cytotoxicity in PBL.

Sham photochemotherapy treatment experiments were conducted with peripheral blood lymphocytes from normal blood bank volunteers. These experiments enabled variation of the intensity of UVA light, the concentration of 8-MOP, and the interaction between 8-MOP and UVA light to be studied in an attempt to elucidate the mechanism of lymphocytotoxicity.

Viability:

Control experiments revealed that 8-MOP alone (without UV irradiation) was not toxic to PBL: 10-300 ng/ml did not affect corrected viability after 72 hours incubation at 37°C. The effect on lymphocyte viability of a wide range of doses of UVA light (0.1-10 J/cm^2) was studied: the results for 1 and 2 J/cm^2 (the dose given to patients) are shown in figure 2A. The addition of 8-MOP clearly potentiated the cytotoxicity caused by UVA light; however, the potentiating effect was dependent on the dose of UVA light. At doses of 3 J/cm^2 and above there was minimal potentiation (data not shown), while at 1-2 J/cm^2 the potentiation was marked. 8-MOP concentrations higher than 50 ng/ml did not further increase cytotoxicity in PBL (data not shown). The viability of lymphocytes up to 96 hr after ECPC is summarized in Table 2.

Table 2

Effect of Extracorporeal Photochemotherapy on Lymphocyte Viability

Lymphocyte sample	% control viability	S.E.
Control	100	5
24 hours post-ECPC	56	9
48 hours post-ECPC	30	5
96 hours post-ECPC	3	1

Samples were taken from patients' buffy coat collections before (control) and after photoirradiation, placed in RPMI 1640 plus 10% fetal calf serum, and trypan blue viability measured for the next 96 hours. The results shown represent the mean of nine patient samples from four patients. The mean control cell viability after exposure to 8-MOP and processing was 85%.

Many cytotoxic agents that are thought to mediate their cytotoxic effect via poly (ADP-ribosyl)ation have their cytotoxicity reduced (or completely eliminated) by the addition of specific poly (ADP-ribose) synthetase inhibitors. The addition of 3-aminobenzamide (ABA) at 2 mM, a dose sufficient to inhibit poly (ADP-ribose) synthetase but which did not affect lymphocyte viability, decreased the cytotoxic effect of exposing PBL to 2 J/cm^2 UVA and 300 ng/ml 8-MOP. This dose of 8-MOP was chosen to maintain an excess of the drug. The viability of the 3-ABA and UVA/8-MOP treated cells (99% at 48 hours) was significantly different from the control UVA/8-MOP group (61%, $p<0.001$) (figure 2B). The protective effect occurred whether the 3-ABA was added before or after irradiation; this excludes the possibility that 3-ABA mitigated the cytotoxic effect merely by decreasing the dose of UVA received by the PBL.

DNA damage:

The DNA damage for a range of UVA light doses (1-5 J/cm^2) was studied 2 hours after irradiation (figure 3). The DNA damaging effect of UVA light on PBL was potentiated by the addition of 8-MOP. However, the extent of this potentiating effect was dependent on the dose of UVA light and the timing of the assay. Edelson et al (1987) reported that the dose of UVA light delivered to their patients' buffy coat lymphocytes was 1-2 J/cm^2; we have shown that in vitro this was the dose where 8-MOP maximally potentiated the DNA damage caused by UVA light. In the sham experiments this dose induced a level of DNA strand breaks similar in extent to those in the patients' samples. At 1.5 J/cm^2 8-MOP increased the number of DNA SSBs by more than twofold. There was far less damage at 24 hours (<15% increase in DNA SSBs) than there was at 2 hours, presumably reflecting DNA repair (data not shown). 5 J/cm^2 of UVA light caused only slightly more DNA SSBs than 2 J/cm^2: this suggests a ceiling effect.

Nucleotide content:

Exposure of PBL to 1-40 J/cm^2 UVA light alone caused a non-linear dose-dependent depletion of NAD and ATP 2 hours after exposure (figure 4A). Between 1 and 10 J/cm^2 there was a steep increase in nucleotide depletion, with further depletion only occurring at very high doses of UVA light. 8-MOP potentiated the NAD depleting effects of UVA light alone. A 1 J/cm^2 UVA light exposure depleted PBL of 11% of their NAD content; adding 8-MOP (300 ng/ml) increased the total NAD depletion to 34% (representative experiment, mean of 5 observations). At higher UVA doses (2-10 J/cm^2) this potentiating effect was not apparent (data not shown). The addition of 8-MOP to 1-2 J/cm^2 UVA light did not further increase ATP depletion.

2 mM 3-ABA also mitigated the nucleotide depletion caused by 10 J/cm^2 of UVA light and continuous exposure to 300 ng/ml 8-MOP (figure 4B). This dose of UVA light was chosen because it caused substantial NAD and ATP depletion; therefore, the mitigating effect of 3-ABA could be clearly demonstrated.

Poly (ADP-ribose) synthetase activity:

PBL were exposed to 10 J/cm^2 UVA light in the presence of 300 ng/ml 8-MOP; at 2 hours poly (ADP-ribose) synthetase activity rose to 160% of control (3 experiments of 3-5 samples). Significant elevations in enzyme activity were not demonstrable with lower doses of UVA light. 8-MOP alone (300 ng/ml) did not affect poly (ADP-ribose) synthetase activity two hours after exposure. 10 J/cm^2 UVA light alone raised enzyme activity to 156% of control (2 experiments of 3 samples). Control lymphocyte poly (ADP-ribose) synthetase activity was 1.20 pmol/min^{-1}/10^6 cells.

Although UVA light caused DNA strand breaks on its own, there was clear evidence that 8-MOP can potentiate the damage caused by UVA light. The degree of this potentiation was highly dependent on the dose of UVA light. The maximum potentiation of DNA damage by 8-MOP occurred at

1.5 J/cm^2. Our observations provide the in vitro rationale for using 1-2 J/cm^2 as the dose of UVA light delivered to buffy coat lymphocytes in the clinical situation. It is also noteworthy that, even with lethal doses (2 J/cm^2) of UVA light and 8-MOP, the DNA damage present at 2 hours was much greater than that at 24 hours after exposure. It appears that this early DNA damage caused a series of biochemical events that irreversibly damaged the cell and that subsequent DNA repair did not affect this.

This UVA/8-MOP-associated DNA damage is associated with adenine nucleotide depletion; our results also suggest that stimulation of poly (ADP-ribosyl)ation and consequent nucleotide depletion are involved in the lymphocytotoxic effect of photoactivated psoralens. Photoirradiation in the presence of 8-MOP caused significantly elevated levels of poly (ADP-ribose) synthetase activity. However, elevated enzyme activity levels were only found at high doses (10 J/cm^2) of UVA light. This may be due to the insensitivity of the assay or may reflect the transient nature of the rise in enzyme activity. The poly (ADP-ribose) synthetase inhibitor, 3-ABA, mitigated the cytotoxicity of photoirradiation and reduced the nucleotide depletion it caused. These three pieces of evidence are regarded as important support for the involvement of poly (ADP-ribosyl)ation (11). Poly (ADP-ribosyl)ation accounts for the majority of NAD turnover in the lymphocyte: it would appear to be the most likely cause for the changes in NAD content that we have demonstrated.

Comparison between photochemotherapy and deoxyadenosine lymphocytotoxicity:

Deoxycoformycin and deoxyadenosine lymphocytotoxicity has been previously shown to be associated with DNA damage and adenine nucleotide depletion, this is thought to be secondary to stimulation of poly (ADP-ribosyl)ation (Althans and Richter, 1987). A 4 hour exposure of PBL to 1 μM dCF and 10 μM dAdo caused 85% cell death in 72 hours (figure 5A), comparable to the delayed

lymphocytotoxicity caused by ECPC. Two hours following drug exposure there was a 19% increase in DNA SSBs. At this time ATP was 66% of control levels and NAD 76% of control (figure 5B): this is compared to the ex vivo effects of ECPC on lymphocytes from CTCL patients. dCF and dAdo caused comparable ATP depletion, but a less marked decrease in NAD.

Our in vitro studies in which normal PBL were treated with dCF and dAdo showed many similarities with the effects of ECPC on lymphocytes of patients with CTCL. The rate of cell kill, DNA strand breakage and extent of NAD and ATP depletion were all similar. Carson et al (1986) originally demonstrated that dAdo and dCF caused DNA strand breaks, depleted cells of NAD and ATP, and increased levels of poly (ADP-ribose) synthesis. They found that nicotinamide and 3-aminobenzamide protected against these changes. We have now shown that photoirradiation is also apparently associated with stimulation of poly (ADP-ribosyl)ation. Although 3-ABA can prevent adenine nucleotide depletion, it appears that the biochemical damage to cells is irreversible (Ganeshaguru et al, 1989). Thus the mechanisms of cell damage caused by these two treatments appear to be closely similar, if not identical.

Example 4

Sensitivity of NOD/WEHI mouse splenocytes to UVA light and 8-methoxypsoralen.

Freshly harvested mouse splenocytes were exposed to UVA light with or without 100 ng/ml 8-MOP and the viability measured by trypan blue exclusion. 1 J/cm^2 UVA in the presence of 100 ng/ml 8-MOP caused 100% cell death in 48 hours (figure 1A).

Sensitivity of NOD/WEHI mouse splenocytes to cytotoxic agents.

The doses of cytotoxic drugs that reproduced the rate of decline in viability caused by exposing mouse splenocytes to 1 J/cm^2 UVA in the presence of 100 ng/ml 8-MOP were identified. Mouse splenocytes were found to be

highly sensitive to low doses of cytotoxic agents.

Various cytotoxic agents that had been dissolved in culture medium were added, and the cells were incubated for various exposure times at 37°C. After washing and resuspension in fresh medium, viability was recorded for the next 48 hours. The pattern of decline of viability caused by UVA/8-MOP was mimicked by dCF/dAdo and by other DNA-damaging drugs (Figure 6B-D).

Apoptotic changes in treated splenocytes.

With human PBL, we showed that either UVA/8-MOP or dCF/dAdo caused apoptotic cell death (Example 2). Accordingly, experiments were conducted in which mouse splenocytes were exposed to either 1 J/cm^2 UVA in the presence of 100 ng/ml 8-MOP, or 1 μM dCF and 10 μM dAdo for 4 hours; 36 hours later, cells were examined by light microscopy for evidence of the morphological changes characteristic of apoptosis.

Control mouse splenocytes contained 12.4% apoptotic cells: the process of cell harvesting and incubation caused a degree of cell damage. UVA/8-MOP-treated cells contained 29.0% apoptotic cells ($p<0.001$), while 17.4% of dCF/dAdo- treated cells were apoptotic 36 hours after exposure ($p=0.09$). Apoptotic cells were identified by the crescentic margination of their nuclear material to the periphery of the nucleus. The size of apoptotic cells appeared reduced, but the plasma membrane remained intact.

Example 5

Prevention of diabetes in NOD/WEHI mice by cytotoxic-treated syngeneic mouse splenocytes.

On day 1, recipient mice were given 350 mg/kg intraperitoneal CP. Retroorbital venous blood was sampled between 14 and 18 days later, and the plasma glucose measured. Recipient mice were divided into 5 groups, of which two were control groups. One of the control groups was given no further treatment, in order to determine the control incidence of CP-induced diabetes; th other was given untreated mouse splenocytes. Untreated cells were

processed identically to the cells that were treated with cytotoxic agents, with the same number of centrifugations and washes. The other 3 groups of mice (C-E) received mouse splenocytes treated with 1 J/cm^2 UVA, 1 J/cm^2 UVA plus 100 ng/ml 8-MOP, or a 4 hour exposure to 1 μM dCF and 10 μM dAdo respectively. Mouse splenocytes were injected immediately after cytotoxic exposure.

There were 139 evaluable mice. Groups were well matched except group E (dCF/dAdo) mice, who received fewer cells than the other 4 groups. There were only small differences between the groups with respect to the ages and initial plasma glucoses of recipient mice. These results are summarized in Table 3.

TABLE 3: Results of infusion of syngeneic mouse splenocytes

	A	B	C	D	E
Number of mice	44	34	15	23	23
Mean age (days)	106	97	93	102	110
Mean number of viable cells infused (x 10^6)	54.8	53.2	56.4	57.7	48.9
Number of diabetic mice at days 14-21 (%)	27 (68)	14 (48)	4 (27)	2 (10)	3 (14)
Number of dead mice	4	4	0	3	0

All groups of mice received 350 mg/kg CP intraperitoneally on day 1. Splenocytes were injected intravenously at 8, 24 and 48 hours.

A. No further treatment.

B. Untreated mouse splenocytes.

C. Mouse splenocytes treated with 1 J/cm^2 UVA only.

D. Mouse splenocytes treated with 1 J/cm^2 UVA in the presence of 100 ng/ml 8-MOP.

E. Mouse splenocytes exposed to 1 μM dCF and 10 μM dAdo for 4 hours.

Chi-squared analysis showed that groups D and E had a significantly lower incidence of diabetes than control group B ($p<0.01$).

A small number of mice (11/139, 8%) died before the 14-18 day plasma glucose could be measured (Table 3). Mice were examined daily, and overtly unwell mice had their glucose measured. Our previous experience has shown that CP 350 mg/kg is associated with a low but definite mortality rate. These mice did not reach the defined endpoint, and have been excluded from further analysis. No treatment group had significantly more deaths.

The percentage (68%) of mice who developed diabetes in the control (no further treatment) group is consistent with previous reports (Charlton et al, 1989). The incidence (48%) in the group that received untreated cells was not significantly less ($p>0.05$). The infusion of UVA/8-MOP-treated splenocytes reduced the incidence to 10% at 14-18 days ($p<0.01$). Further glucose measurements at 21 and 28 days showed that diabetes was prevented, and not just delayed. The UVA-only-treated splenocyte group had more diabetes (4/15, 27%), but this was not statistically significantly different to the incidence in the UVA/8-MOP group (2/20, 10%, $p>0.10$).

There were 23 mice in the dCF/dAdo-treated splenocyte group; all were evaluable. This treatment reduced the incidence of diabetes to 14% ($p<0.01$), and appeared to have the same efficacy as UVA/8-MOP ($p>0.50$). It is noteworthy that this group actually received fewer splenocytes than the other treatment groups.

In this study, donor splenocytes from CP-induced diabetic NOD/WEHI mice, which are activated against pancreatic beta cells, were lethally damaged by UVA/8-MOP. Infusion of these "programmed-to-die" splenocytes prevented diabetes in syngeneic mice. We postulate that UVA/8-MOP

damage results in the splenocytes being recognised by normal (recipient mouse) T-lymphocytes. Presumably, these cells then, by an as yet undetermined mechanism, decrease the quantity and function of the recipient mouse lymphocytes that infiltrate islets and cause diabetes. There is evidence from a murine skin allograft model that UVA/8-MOP treatment of autoreactive T-cells, followed by the reintroduction of these cells, can lead to a clone-specific suppressor cell response that downregulates abnormal T-cell activity (Perez et al).

Thus we have now found that dCF/dAdo, as well as mimicking the in vitro biochemical and physical effects of UVA/8-MOP treatment of human peripheral blood lymphocytes, causes similar effects in an in vivo murine autoimmune disease model. Although the two cytotoxic agents have different primary mechanisms of action, it seems likely that their ability to damage the DNA of lymphocytes results in a common form of programmed cell death, presumably inducing a common immunological effect.

We have also shown that UVA/8-MOP and dCF/dAdo treatment of human PBL cause

(a) a similar pattern of lymphocyte cytotoxicity with time,

(b) DNA strand breakage, and

(c) stimulation of poly (ADP-ribosyl)ation with rapid induction of NAD and ATP depletion.

This process is followed by morphological changes of apoptosis and endonucleolytic DNA cleavage with a characteristic "nucleosomal ladder" pattern of fragments that are multiples of 180 base pairs. Similar morphological changes characteristic of apoptosis were seen in the mouse splenocytes. A single dose of cells may be sufficient; the dCF/dAdo group received fewer cells, but this reduced dose was not any less efficacious.

Thus, the mechanism of the immunological effect of the in vitro syngeneic splenocytes may be specifically related to changes in those apoptotic cells. It is not known whether this reflects nuclear, membrane or cytoplasmic changes in the infused cells.

In studies using spontaneously diabetic NOD/Lt mice, we have also shown that diabetes can be prevented using weekly infusions of UVA/8-MOP- treated splenocytes. These mice are not treated with CP, suggesting that this drug does not affect the response of NOD mice to the infused cells.

It will be clearly understood that the invention in its general aspects is not limited to the specific details referred to hereinabove.

References cited herein are listed on the following pages.

REFERENCES

Althaus, F.R., and C.Richter
ADP-ribosylation of proteins. Enzymology and biological significance.
Mol. Biol. Biochem. Biophys. 1987, 37: 1-237

Anderson, T.F., and J.J.Voorhees.
Psoralen photochemotherapy of cutaneous disorders. Ann. Rev. Pharmacol. Toxicol. 1980, 20: 235-237.

Bach J-F., and T.B.Strom.
Alkylating agents.
The Mode of Action of Immunosuppressive Agents. 2nd ed. Amsterdam, 1985 Elsevier, pp. 175-239.

Berger C.L.
Experimental murine and primate models for dissection of the immunosuppressive potential of photochemotherapy in autoimmune disease and transplantation.
Yale J. Biol. Med. 1989, 62:611-620.

Berger, N.A. Symposium: cellular response to DNA damage: the role of poly (ADP-ribose).
Radiat. Res. 1985, 101: 4-15.

Berger, N.A., G.Weber, and A.S.Kaichi. Characterisation and comparison of poly (adenosine diphosphoribose) synthesis and DNA synthesis in nucleotide-permeable cells.

Biochim. Biophys. Acta. 1978, 519: 87-104.

Birnboim, H.C., and J.J.Jevcak.
Fluorometric method for rapid detection of DNA strand breaks in human white blood cells produced by low doses of radiation.
Cancer Res. 1981, 41: 1889-1892.

Carson, D.A., S.Seto, D.B.Wasson, and C.J.Carrera.
DNA strand breaks, NAD metabolism and programmed cell death. Exp. Cell Res. 1986, 164: 273-281.

Charlton B., A.Bacelj, R.M.Slattery, and T.E.Mandel.
Cyclophosphamide-induced diabetes in NOD/WEHI mice.
Diabetes 1989, 38:441-447.

Cohen, I.R. and H.L. Weiner
T Cell Vaccination
Immunology Today 1988, 9: 332-335

Crescentini, G., and V.Stocchi.
Fast reversed phase high performance liquid chromatographic determination of nucleotides in red blood cells.
J. Chromatog. 1984, 290: 393-399.

Edelson R., C.Berger, F.Gasparro, B.Jegasothy, P.Heald, B.Wintroub, R.Knobler, K.Wolff, G.Plewig, G.McKiernan, I.Christiansen, M.Osler, H.Honigsmann, H.Wilford, E.Kokoschka, T.Rehle, M.Perez, G.Stingl, and L.Laroche.
Treatment of cutaneous T-cell lymphoma by extracorporeal photochemotherapy.
N. Engl. J. Med. 1987, 316:297-303.

Ganeshaguru, K., A.Piga, L.Latini, and A.V.Hoffbrand.
Inability of poly-ADP-ribosylation inhibitors to protect peripheral blood lymphocytes from the toxic effects of ADA inhibitors.
Adv. Exp. Med. Biol. 1989, 253B: 251-258.

Gilchrest, B.A., J.Parrish, L.Tanenbaum, H.A.Haynes, and T.B.Fitzpatrick.
Oral methoxsalen photochemotherapy of mycosis fungoides.
Cancer 1976, 38: 683-689.

Harada M., and S.Makino.
Promotion of spontaneous diabetes in non-obese diabetes-prone mice by cyclophosphamide. 1984.
Diabetologia 1984, 27:604-606.

Knobler, R.M., and R.L.Edelson.
Cutaneous T-cell lymphoma.
Med. Clin. N. Amer. 1986, 70: 109-138.

Makino S., K.Kunimoto, T.Muraoka, Y.Mizushima, K.Katagiri, and Y.Tochino.
Breeding of a non-obese diabetic strain of mice.
Exp. Anim. (Tokyo) 1980, 29:1-13.

Perez M., R.Edelson, L.Laroche, and C.Berger.
Inhibition of antiskin allograft immunity by infusions with syngeneic photoinactivated effector lymphocytes. J. Invest. Dermatol. 1989, 92:669-676.

Puglisi, C.V., A.J.de Silva, and J.C.Meyer.
Determination of 8-methoxypsoralen, a photoactive compound, in blood by high pressure liquid chromatography.
Anal. Lett. 1977, 10: 39-50.

Rockman, S.P., E.J.Wilde, M.A.Oziemski, G.Varigos, M.D.Green, and R.M.Fox. 1989
Immunological changes in patients receiving 8-methoxypsoralen and extracorporeal photochemotherapy for cutaneous T-cell lymphoma.
Haematology Society of Australia meeting, Adelaide. (Abstr.)

Rook A.H., B.Freundlich, G.T.Nahass, R.Washko, B.Macelis, M.Skolnicki, P.Bromley, W.K.Witmer, and B.V.Jegasothy.
Treatment of autoimmune disease with extracorporeal photochemotherapy: progressive systemic sclerosis.
Yale J. Biol. Med. 1989, 62:639-645.

Yasanumi R., and J-F.Bach.
Anti-suppressor effect of cyclophosphamide on the development of spontaneous diabetes in NOD mice.
Eur. J. Immunol. 1988, 18:481-484.

CLAIMS

1. A pharmaceutical preparation for specifically altering the immune response of a mammal, comprising mononuclear leucocytes from said mammal, which leucocytes have been treated by extracorporeal incubation with an agent which induces irreversible damage to the cells or cell death.

2. A pharmaceutical preparation according to Claim 1, wherein the mononuclear leucocytes to be treated by extracorporeal incubation are obtained from blood, lymph, bone marrow, or lymphoid tissues, and are either normal, or activated *in vivo* or *in vitro*.

3. A pharmaceutical preparation according to Claim 1 or Claim 2 wherein the cells to be treated by extracorporeal incubation are lymphoid cells.

4. A pharmaceutical composition for treatment of an immunologically-mediated condition or a cancer, comprising an effective amount of a preparation as defined in any one of Claims 1, 2, or 3, together with a pharmaceutically acceptable carrier or diluent.

5. A method for prevention or treatment of an immunologically-mediated condition or of a cancer, comprising the step of administering to a patient in need of such treatment an effective amount of a composition as defined in Claim 4.

6. A method of preparation of a pharmaceutical preparation according to any one of Claims 1, 2, or 3, comprising the step of incubating a sample of cells from a patient suffering from an immunologically-mediated condition or a cancer, said sample comprising normal or malignant mononuclear leucocytes, with an agent which induces irreversible damage to the cells or cell death, for a period and under conditions effective to cause irreversible damage to said cells, and optionally washing the cells.

7. A method according to Claim 6, wherein the agent induces cellular DNA damage and is selected from the group consisting of compounds with cytotoxic activity, antineoplastic activity, immunosuppressive activity, or anti-viral

activity.

8. A method of preparation of a composition according to Claim 6, wherein the agent is selected from the group consisting of;

(a) nucleoside analogues,
(b) alkylating agents,
(c) antimetabolites,
(d) inhibitors of topoisomerase II,
(e) antibiotics which induce DNA strand breakage, and
(f) steroids.

9. A method according to Claim 8, wherein the nucleoside analogue is selected from the group consisting of adenosine analogues, 2'-deoxyadenosine, 3'-deoxyadenosine (cordycepin), tubercidin and 9-(β-D-arabinofuranosyl)- adenine (Ara-A; vidarabine).

10. A method according to Claim 8, wherein the alkylating agent is mafosfamide or 4-hydroperoxy-cyclophosphamide.

11. A method according to Claim 8, wherein the antimetabolite is methotrexate.

12. A method according to Claim 8, wherein the inhibitor of topoisomerase II is etoposide (VP-16.213), teniposide (VP-16), or another derivative of podophyllotoxin.

13. A method according to Claim 8, wherein the antibiotic is selected from the group consisting of bleomycins, mitomycin, anthracyclines and gliotoxin.

14. A method according to Claim 6 wherein an alkylating agent is used in combination with an anti-metabolite.

15. A method according to Claim 6 wherein an adenosine analogue is used in combination with an adenosine deaminase inhibitor.

16. A method according to Claim 10 wherein the adenosine deaminase inhibitor is selected from the group consisting of deoxycoformycin, erythro-9-(3-hydroxy-2-nonyl) adenine, and 2-chlorodeoxyadenosine.

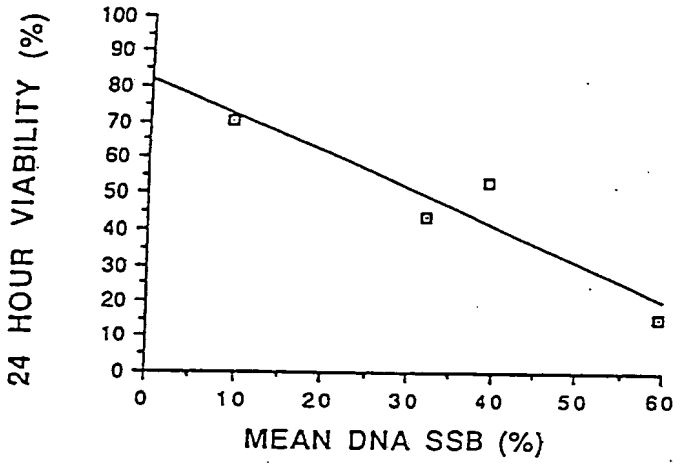


FIGURE 1

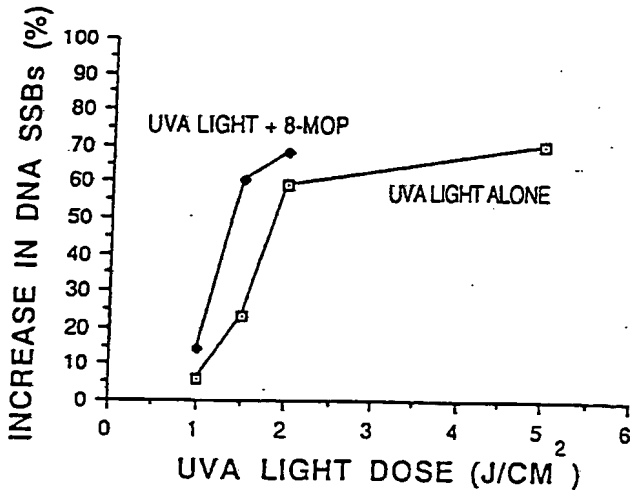


FIGURE 3

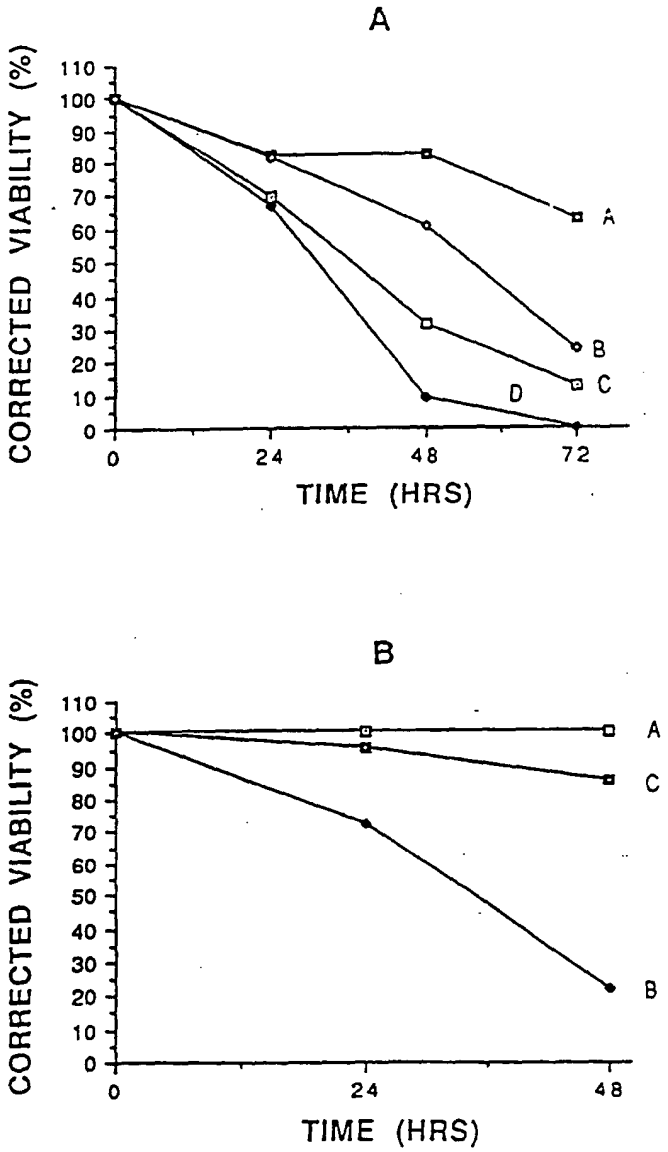
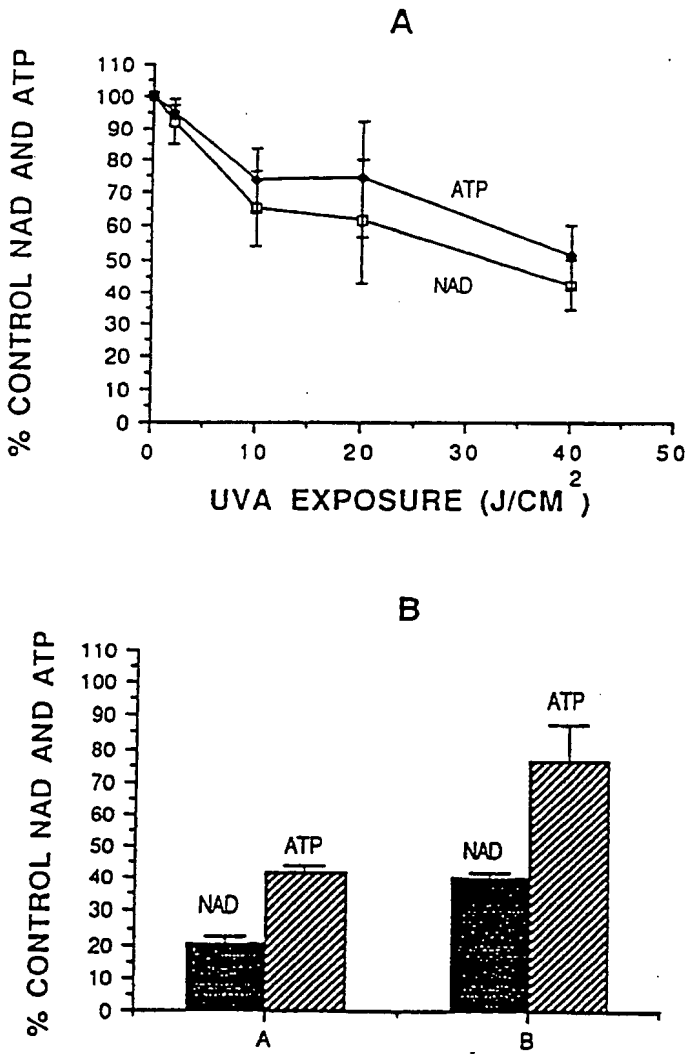

A
CORRECTED VIABILITY (%)
110
100
90
80
70
60
50
40
30
20
10
0
0
24
48
72
TIME (HRS)
A
B
C
D
B
CORRECTED VIABILITY (%)
110
100
90
80
70
60
50
40
30
20
10
0
0
24
48
TIME (HRS)
A
C
B

FIGURE 2


A
% CONTROL NAD AND ATP
110
100
90
80
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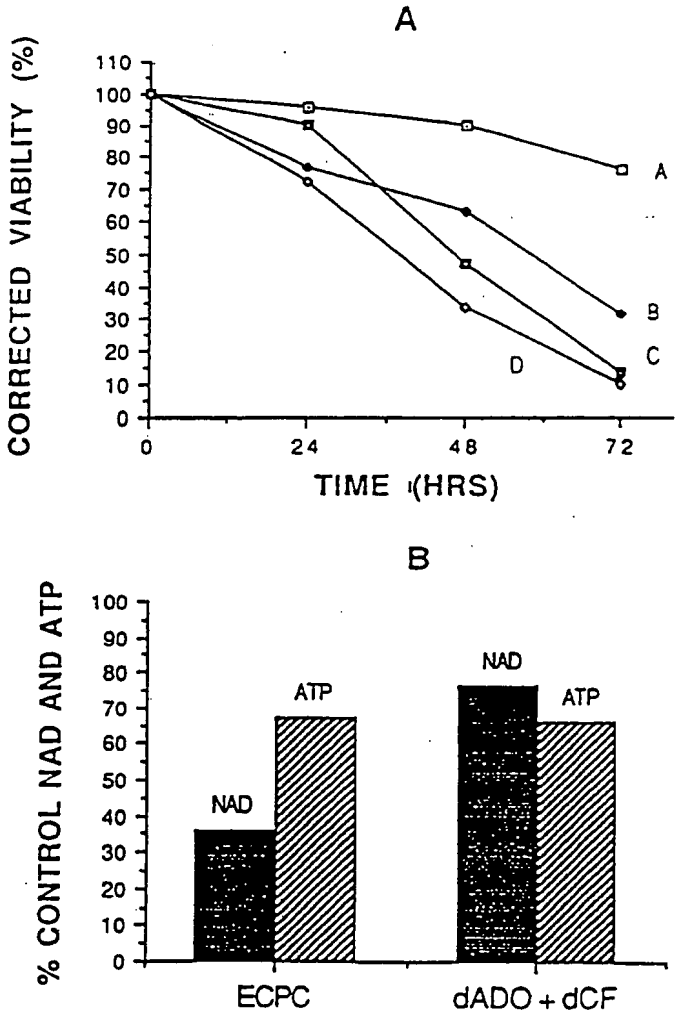
40
50
ATP
NAD
UVA EXPOSURE (J/CM 2)
B
% CONTROL NAD AND ATP
NAD
ATP
NAD
ATP
A
B

FIGURE 4


A
CORRECTED VIABILITY (%)
110
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90
80
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24
48
72
TIME (HRS)
A
B
C
D
B
% CONTROL NAD AND ATP
100
90
80
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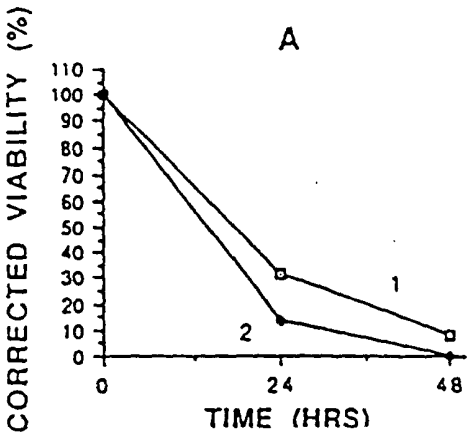
60
50
40
30
20
10
0
NAD
ATP
NAD
ATP
ECPC
dADO + dCF

FIGURE 5


A
CORRECTED VIABILITY (%)
110
100
90
80
70
60
50
40
30
20
10
0
1
2
0
24
48
TIME (HRS)

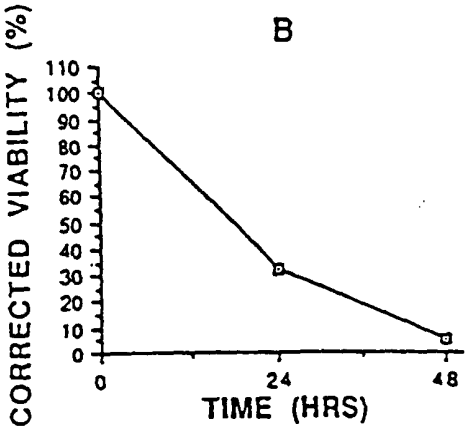
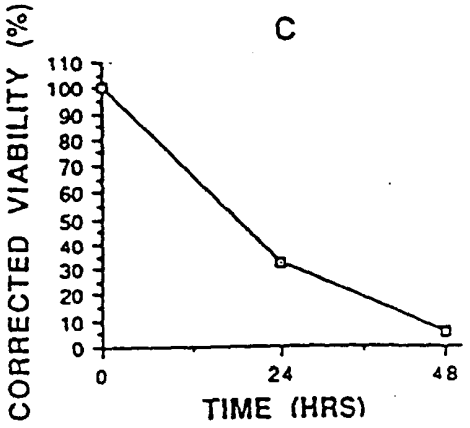

B
CORRECTED VIABILITY (%)
110
100
90
80
70
60
50
40
30
20
10
0
0
24
48
TIME (HRS)

FIGURE 6A


C
CORRECTED VIABILITY (%)
110
100
90
80
70
60
50
40
30
20
10
0
0
24
48
TIME (HRS)

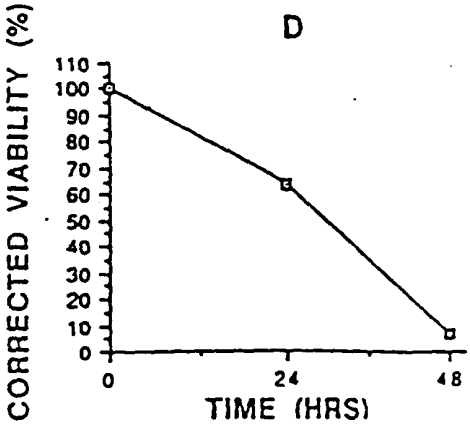

D
CORRECTED VIABILITY (%)
110
100
90
80
70
60
50
40
30
20
10
0
0
24
48
TIME (HRS)

FIGURE 6B

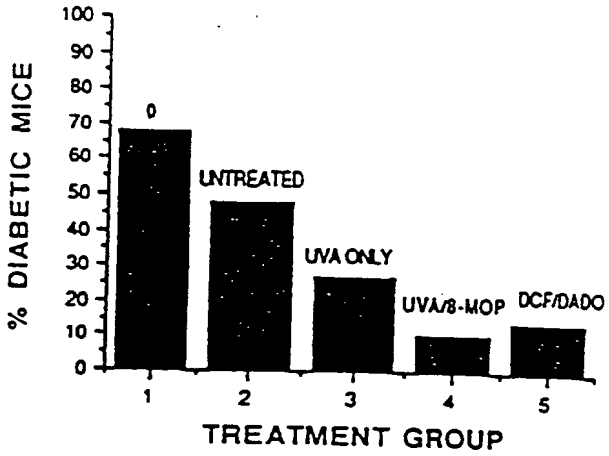

% DIABETIC MICE
100
90
80
70
60
50
40
30
20
10
0
0
UNTREATED
UVA ONLY
UVA/8-MOP
DCF/DADO
1
2
3
4
5
TREATMENT GROUP

FIGURE 7

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent classification (IPC) or to both National Classification and IPC

Int. Cl.[5] A61K 35/14, 35/26, 45/05

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	A61K 35/14, 35/26, 45/05
CHEM. ABS.	KEYWORDS; LEUKOCYTE AND 2-DEOXYADENOSINE, CORDYCEPIN, ETOPOSIDE, METHOTREXATE, METHOXSALEN, PENTOSTATIN

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

DIALOG; BOISIS, MEDLINE KEYWORDS; LEUKOCYTE AND 2-DEOXYADENOSINE, METHOTREXATE, ETOPOSIDE

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category*	Citation of Document, [11] with indication, where appropriate of the relevant passages [12]	Relevant to Claim No [13]
X	US,A, 4428744 (EDELSON) 31 January 1984 (31.01.84), see whole document.	(1-6)
X	GB,A, 2048069 (LIMBURG) 10 December 1980 (10.12.80), see Table 1 and claims 1-14.	(1-8, 10-11)
X	GB,A, 2012584 (JAEGER) 1 August 1979 (01.08.79), see page 3 lines 23-29 and claims 1-9.	(1-8, 13)
A	US,A, 4844893 (HONSIK et al) 4 July 1989 (04.07.89), see whole specification	(1-6)

* Special categories of cited documents : [10]

"A" Document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior t the international filing date but later than the priority date claimed

"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 March 1992 (17.03.92)	26 March 1992 (26.03.92)
International Searching Authority	Signature of Authorized Officer
AUSTRALIAN PATENT OFFICE	J.H. CHAN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, [11] with indication, where appropriate of the relevant passages [12]	Relevant to Claim No [13]

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4a

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ *As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.*

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is *restricted to the invention first mentioned in the claims; it is covered by claim numbers:*

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ *The additional search fees were accompanied by applicant's protest.*

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL APPLICATION NO. PCT/AU 91/00563

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-m ntioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member		
US 4428744	AU 65171/80	BR 8008053	CA 1153334
	DK 5200/80	EP 30358	ES 497578
	ES 8202484	JP 56131520	NO 803706
	US 4321919	ZA 8007529	US 4398906
	US 4428744	US 4464166	US 4612007
	US 4613322	US 4683889	US 4684521
	AU 83824/82	BE 893481	BR 8203384
	CA 1176165	DE 3222244	ES 513014
	ES 8400669	FR 2507482	GB 2100143
	IT 1151636	JP 57211354	SE 8203547
	ZA 8203589	CA 1235191	EP 111418
GB 2048069	AT 2402/80	AU 58069/80	CA 1139219
	CH 647152	DE 2918927	DK 1895/80
	EP 19167	ES 491356	FI 801420
	FR 2455891	GB 2048069	IT 1133086
	JP 55151515	NO 801388	PL 224116
	PT 71195	ZA 8002391	
GB 2012584	AT 267/79	BE 873544	DE 2802277
	DK 227/79	ES 477013	FI 790150
	FR 2414919	GB 2012584	GR 65262
	IT 1111003	JP 54113411	NL 7900365
	NO 790177	PT 69100	SE 7900421
	ZA 7900221		
US 4844893	AU 81089/87	DK 2746/88	EP 287615
	FI 882668	NO 882494	US 4844893
	WO 8802773		